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DNA vaccination in mice using HIV-1 nef, rev and tat genes in self-replicating pBN-vector

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Abstract

The immunogenicity of a self-replicating DNA-vector containing HIV-1 nef gene (pBN-Nef) was characterized using various DNA delivery methods. In addition, gene gun immunisation was used for assessing immunogenicity of two other HIV-1 genes (rev and tat) given in the same vector. The pBN-Nef was the most immunogenic raising both humoral and cell-mediated immune responses in mice; these responses lasted for up to six months. The pBN-Nef vector was immunogenic also when given intramuscularly or intradermally. The pBN-Rev construct did not elicit humoral responses but did elicit proliferative as well as CTL-response against the corresponding protein. The pBN-Tat was a poor immunogen in all respects. The antibodies elicited with various DNA delivery methods belonged to different antibody subclasses; however, two main epitopes in Nef were frequently recognized by all of them.

Keywords: DNA vaccination; HIV-1; Nef; Rev; Tat

1. Introduction

Since the first DNA immunisations showed that such a method is a safe and potent vaccination strategy capable of inducing both humoral and cell-mediated immune response, several experimental DNA prototype vaccines have also been developed for HIV-1. These include DNA constructs containing genes for HIV structural proteins [1–4] as well as for regulatory or accessory proteins [5–8]. Various DNA delivery methods [9,10] and also animal species including non-human primates and humans have already been tested [1–4,6].

The regulatory proteins Rev and Tat as well as the accessory protein Nef belong to proteins expressed early in the life cycle of the virus, soon after virion entry into the cell [11]. Thus, an immune response directed against them may prevent later steps in HIV life cycle and inhibit the release of progeny virus particles. In fact, an immune response against these early proteins has been shown to correlate with protection or attenuation of the disease [12–16]. In order to find an efficient DNA-vaccine based on these early proteins, we recently developed novel DNA constructs containing regulatory elements from bovine papilloma virus (BPV), and showed that a self-replicating pBN-Nef vector is capable of inducing both humoral and cell-mediated immune responses [17].

In this study we have further evaluated the applicability of this vector by looking at its immunogenicity using different DNA delivery methods (e.g. gene gun delivery, intramuscular or intradermal immunisation). In addition, the immunogenicity of two other HIV-1

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genes (rev and tat) given in pBN-vectors were characterized. These genes, which have many effects on virus replication and on the function of infected cells, are potent candidates for vaccine and therapeutic development. For efficacy evaluation, antigen-specific antibody production as well as CTL- and proliferative-responses were measured. Also, to assess the Th-type of the response, antibody subclasses in mice immunised by various methods were analyzed and Nef-specific epitopes recognized by these antibodies were characterized.

2. Materials and methods

2.1. Plasmids

The pBN-vector which has the immunogenic foreign gene cloned under RSV-promoter [17] was used for expression of nef, rev and tat genes. The cDNAs of these genes (isolate BRU) were amplified with polymerase chain reaction (PCR) from the corresponding pc-plasmids (a generous gift from Dr Brian Cullen, Howard Hughes Medical Institute, Duke University Medical Center, USA) and cloned into a pUE83 shuttle vector (Tartu University). A *Hin*dIII cassette of the shuttle vector containing the BPV-*ori* and RSV promoter was further cloned into pBN-vector (Tartu University) having the BPV-1 derived E1 and E2 genes shown to be necessary and sufficient for transcriptional enhancement and self-replication of BPV-*ori* containing plasmids [18]. The BPV E1 was cloned either under SRα or a TK promoter.

2.2. Confirming protein production in vivo

2.2.1. Transfection and immunohistochemical staining of COS cells

COS-1 cells grown overnight on 6-well plates were transfected with plasmid DNA using commercial FuGene (Boehringer) preparation. The amount of DNA and FuGene reagent per well was 1 μg. In our experience FuGene transfection efficiency determined by counting antigen expressing cells varies between 50–70%. Cells were harvested after 72 h and protein expression was detected by Western blotting and immunohistochemical staining using Nef-, Rev- or Tat-specific monoclonals (Fig. 1).

2.2.2. Detection of expression of functional Rev from rev-encoding plasmids by HIV-1 p24 ELISA

An antibody based ELISA [19] was used to assay the amount of Gag (p24) protein expressed from a Gag-encoding plasmid pNLgagSty330 which carries the rev responsive element (RRE) sequence, the gag gene, HIV-1 5′ and 3′ LTRs as promoter and poly A. Briefly, using FuGene 6TM reagent (Boehringer) HeLa cells were tranfected with the plasmid encoding the rev gene (10 pg, 100 pg, 1 ng, 10 ng, 100 ng or 1 μg) mixed with 0.5 μg pNLgagSty330 plasmid and 0.5 μg of the HCMVtat plasmid [6]. The HCMVrev plasmid [6] recently tested

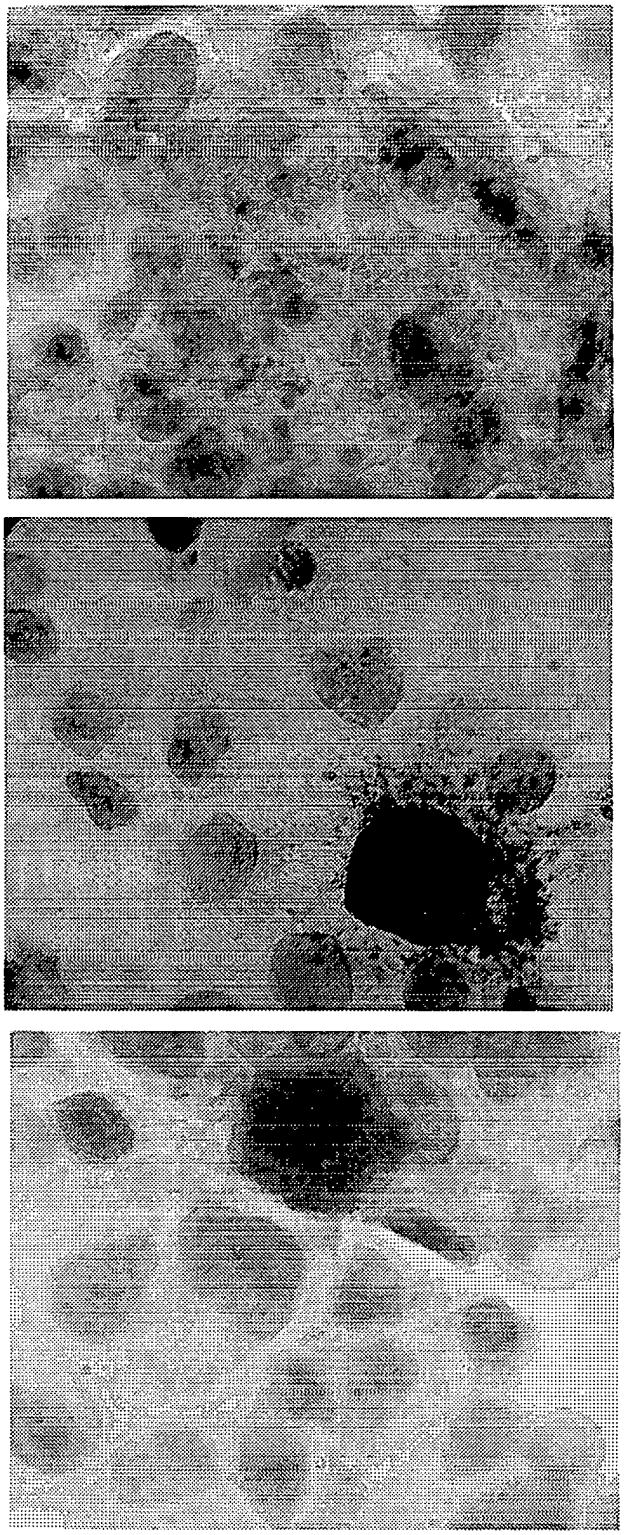

Fig. 1. Immunohistochemical staining of COS-1 cells 72 h after transfection with (a) pBN-Nef, (b) pBN-Rev and (c) pBN-Tat.

in clinical trials was used as a positive control. The total amount of plasmid was kept constant (2 μg) by addition of a non-coding plasmid pBluescript (Stratagene). All transfected plasmids were used alone as controls for unspecific p24 production. The transfected cells were incubated for 24 h and the amount of p24 produced was assayed by an HIV-1 p24 ELISA as previously described [20]. A standard curve was fitted and the concentrations of p24 in the samples were calculated according to linear regression analysis.

2.2.3. Detection of functionally active Rev by cotransfection experiments with SIV (Rev −)

As HIV-1 Rev can transcomplementate SIV Rev [21] in cells infected with Rev-defective SIV leading to abundant virus production, COS-1 cells were cotransfected with pBN-Rev and a Rev-defective SIV construct (total amount of DNA being 2 μg and the ratio of plasmids 3:1, respectively) using the FuGene as described above. Supernatant was harvested 72 h after transfection and assayed for SIV p27 antigen using a SIV Ag kit (Coulter) according to instructions given by the manufacturer.

2.2.4. Detection of Tat expression from tat-encoding plasmids by CAT ELISA

An enzyme based ELISA [19] was used to assay the amount of enzyme expressed from a CAT (chloramphenicol acetyl transferase) encoding plasmid pNLCATw carrying the 5′ and 3′ LTRs from HIV-1. Tat protein binds to the TAR element in the LTR and promotes CAT expression. The plasmid DNA encoding the tat gene (0.01 μg, 0.1 μg, 1 μg) was mixed with 1 μg of the pNLCATw plasmid and HeLa cells were transfected using FuGene transfection reagent. The pNLCATw (1 μg) alone was used as a control for unspecific CAT expression and HCMVtat [6] as a positive control. The amount of transfected DNA was kept constant (2 μg) by addition of a non-coding plasmid (pBluescript) DNA. The transfected cells were incubated 24 h, washed, harvested and lysed. The cell debris was separated from the CAT-containing supernatant by centrifugation.

The amount of CAT expressed was immediately assayed by a CAT ELISA (Boehringer) using standards consisting of recombinant CAT enzyme from *Escherichia coli*. All samples were pipetted onto the anti-CAT-antibody coated microtiter plates, incubated at + 37°C for 1 h, washed and digoxigenin (DIG) labelled anti-CAT antibodies were added to each well for another 1 h incubation. After washings, the plates were incubated with anti-DIG-peroxidase (POD) antibodies, washed again and the colour intensity developed from the POD substrate was measured at 405 nm (reference 490 nm). A standard curve was fitted and the concentrations of CAT produced in the samples were calculated according to linear regression analysis. The assay was repeated a week later in order to detect prolonged expression of Tat.

2.3. Immunisation protocols

Balb/c mice (5–8 weeks old) were used for all immunisation experiments. In four different experiments, a total of 33 mice were immunised with pBN-Nef using a gene gun (Nef GG group); 23 of them were sacrificed 4 weeks after the last immunisation and the rest were followed up by sacrificing two mice per month for up to 6 months. Similarly, a total of 23 mice were immunised with pBN-Rev (Rev GG group) and 23 with pBN-Tat (Tat GG group); 13 mice from each group were sacrificed after 4 weeks and the rest were followed up for 6 months. Four control mice received only the plain vector without HIV-1 genes cloned in. For assessing the immunogenicity of pBN-vectors in different delivery methods, in the last experiment 12 mice received pBN-Nef intramuscularly (Nef IM group) and 12 mice intradermally (Nef ID group) while 12 mice received it using gene gun immunisation.

As 99% of intradermally or intramuscularly immunised DNA is degraded within 90 min [22] and as there are limits in the loading capacity of the gold particles, multiple injections were considered necessary to ensure that each mouse receives adequate amounts of DNA. Furthermore, multiple inoculations (3–6 ×) have been generally used in DNA immunisations [1,2,4,6–8]. Each mouse received six injections within 2 weeks the total amount of DNA administered being 6 μg for gene gun immunisation (GG) and 150 μg for intramuscular (IM) and intradermal immunisation (ID). For gene gun immunisations, plasmids were precipitated onto 1 μm gold particles following the procedure in the Helios gene gun instruction manual (Bio Rad Laboratories). The cartridges contained 0.5 mg gold and 1 μg DNA each. Mice were immunised on shaved abdominal skin using a helium discharge pressure of 300 psi [17]. For intradermal or intramuscular immunisations, plasmid DNA was dissolved in sterile saline (25 μg/20 μl) and injected into the dorsal skin or quadriceps femoris, respectively.

2.4. Humoral immune assays

2.4.1. Detection of antibodies and their subclasses in ELISA assay

The ELISA method used was performed as previously described [17]. As antigen, we used recombinant GST-Nef (kindly provided by Dr Harris and the MRC AIDS Directed Programme Reagent Project), Rev or Tat protein (Intracell, USA) shown in Western blotting and SDS gels to be monomers and devoid of protein contaminants. Peroxidase conjugated anti-mouse IgG (DAKO) or anti-mouse IgG1, IgG2a, IgG2b, IgG3 or

IgM (Caltag) were used as secondary antibodies. An ELISA value (EIU) was calculated using the following formula EIU = ($OD_{mouse\ serum} - OD_{normal\ mouse\ serum}$) × 100/($OD_{positive\ control\ mouse\ serum} - OD_{normal\ mouse\ serum}$). Serum from a mouse immunised with whole protein was used as a positive control. An EIU value above 10 was considered positive. The presence of Nef-specific antibodies was confirmed with Western blotting, and titers showing the highest dilution of sera still positive were determined by ELISA (cut-off: mean of normal mice sera + 3 S.D.). The IgG1/IgG2a ratio for each Nef-antibody positive mouse was calculated from the corresponding OD values measured against whole protein.

2.4.2. Epitope mapping

The detection of antigenic regions of Nef was performed with ELISA as described above. As an antigen, 15–17 mer overlapping peptides scanning the whole amino acid sequence of isolate BRU Nef were used. The peptides were received from European Vaccine against AIDS (EVA) reagent repository. The amount of peptide per well was 500 ng. Three sera from each immunisation group giving high EIU values against whole Nef protein (titers 1:5000–1:10 000) were selected for epitope mapping. Their reactivity against each peptide was indicated with an ELISA value (EIUpep) calculated using the following formula EIUpep = ($OD_{mouse\ serum} - OD_{normal\ mouse\ serum}$) × 100/($OD_{mouse\ serum\ against\ whole\ Nef} - OD_{normal\ mouse\ serum\ against\ whole\ Nef}$). An EIUpep value above 10 was considered positive.

2.5. Cell-mediated immunity assays

2.5.1. Proliferation assay

For the proliferation assays, spleen cell suspensions were prepared from the DNA-immunised and control mice. Cells were cultured for 5 days in RPMI 1640 medium containing 10% FCS + glutamin + antibiotics + 2-mercaptoethanol in 96-well flat bottom plates (200 μl/well). Antigens were added to triplicate wells at the following final concentrations: Concanavalin A 2.5 μg/ml (positive control), recombinant Nef [23], Rev or Tat proteins (Intracell, USA) 1 μg/ml and 10 μg/ml and PPD (negative control) 10 μg/ml. After 5 days culturing proliferative responses were measured with [^{3}H]thymidine incorporation [24]. The stimulation index (SI) was determined as (experimental count − spontaneous count)/spontaneous count. An SI above 3 was considered positive. Due to the lack of purified antigen, the proliferation assay could not be performed for all mice.

2.5.2. CTL assay

Nef, Rev and Tat specific CTL responses were measured as previously described [17] in the ^{51}Cr release assay using mouse effector splenocytes, activated by co-culturing them with antigen presenting cells. For antigen presentation, syngeneic P815 mastocytoma (H-2^d) cells were infected with recombinant Vaccinia virus expressing the HIV-1 LAI nef, rev or tat genes (Transgene, France). Infections with Vaccinia constructs were performed at a multiplicity of infection (MOI) of 5 for 16 h at + 37°C, after which the cells were washed twice with PBS containing 10% fetal calf serum (FCS, GibcoBRL), and γ irradiated at 5000 rad; when used as target cells in CTL assay the cells were not irradiated. The cells were washed with culture medium before adding to the effector cells. An effector-to target ratio of 50:1 was used. The percentage of specific lysis ≥ 6% was considered to be positive as 6% was calculated to be a statistically significant difference compared to control cells infected with wild type vaccinia virus.

3. Results

3.1. Protein expression in transfected cells

When various mammalian cell lines (HeLa, COS-1) were transfected with pBN-plasmids containing the nef, rev or tat genes, protein expression could be detected by Western blotting and immunohistochemical staining (Nef, Rev and Tat; Fig. 1) and by functional assays (Rev, Tat). The functional assay results for plasmids are shown in Fig. 2. Panel 2a shows that Rev-dependent p24 production was detected even when as little as 10 pg of pBN-Rev plasmid was cotransfected with 0.5 μg pNLgagSty330-plasmid into cells. Furthermore, when the amount of transfected DNA was above 10 ng, the amount of p24 induced by pBN-Rev exceeded the amount induced by the positive control HCMVrev plasmid [6]. At lower concentrations these two plasmids were equally efficient (data not shown). Also, the protein produced by pBN-Rev was functionally similar to the native one in the capability to cross-activate the rev-defective SIV for p27 production (Fig. 2b). The fact that the protein expressed by the pBN-Rev plasmid can compensate the function of Rev in a Rev-defective SIV construct proves that the plasmid produces functional Rev. The result of functional assay for pBN-Tat is shown in Fig. 2c: 10 ng of the plasmid cotransfected with the plasmid pNLCATw into HeLa cells was the minimal amount of DNA needed to show activation of CAT production. The efficacy of pBN-Tat in inducing CAT production was below that of the control plasmid HCMVtat [6]. However, when pBN-Tat transfected cells were tested 1 week later, CAT production was still detectable (Fig. 2c).

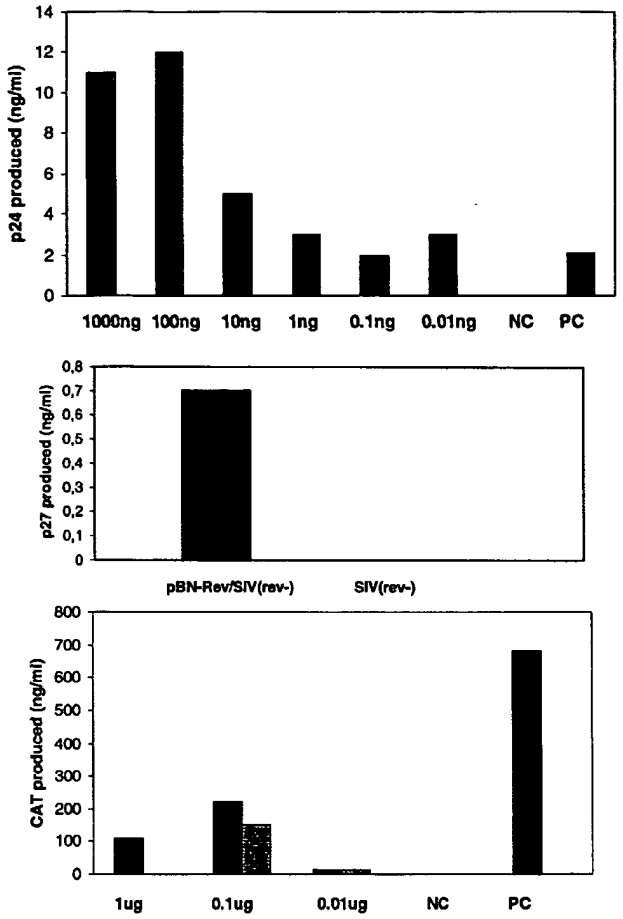

Fig. 2. Functional assays showing the effects of expressed Rev and Tat proteins in pBN-transfected mammalian cells. The total amount of transfected DNA in each assay was kept constant (2 μg). (a) Rev-dependent p24 production from cells cotransfected with Gag-encoding plasmid pNLgagSty330 and various amounts of pBN-Rev, (b) Rev-dependent p27 production from cells cotransfected with pBN-Rev and SIV (rev −) and (c) Tat-dependent CAT production in cells cotransfected with pNLCATw and various amounts of pBN-Tat. Black bars: assay done 24 h after transfection; gray bars: assay done 1 week later. NC: negative control; PC: positive control (shown is the result of transfection using 100 ng of control plasmid).

3.2. Humoral immune response in vaccinated mice

Table 1 shows the immune results of mice immunised with plasmids containing different regulatory genes and using various DNA delivery methods. In Nef GG group all mice developed specific antibodies within 4 weeks after the last immunisation; the EIU values for Nef gene gun immunised mice varied between 25–78 (mean 51). Antibody response could be detected up to 6 months (Table 2) with no decrease in EIU values. In the Nef IM group 50%, and in Nef ID group 75% of mice showed antibodies against this protein (Table 1). EIU values in the IM group varied between 24–75 (mean 43) and in the ID group between 12–100 (mean 56). The titers of Nef-positive sera varied between 1:100–1:10 000, and a clear correlation between titer and EIU value was seen. In the Rev GG and Tat GG groups no antibodies were detected after 4 weeks. The IgG1/IgG2a ratio was much higher in the Nef GG group than in the Nef IM group (Fig. 3) showing that gene gun immunisation raised mainly a Th2 type response distinguished by IgG1 class antibodies [25] and intramuscular immunisation caused a Th1 type response where IgG2a class antibodies prevail. The response in the ID group seemed to have a mixed character. In gene gun immunised mice IgG1 antibody class remained elevated throughout the 6 months follow up period indicating no class switching taking place later on (data not shown). Other antibody subclasses tested were not significantly elevated in any of the Nef groups. None of the control mice had Nef-, Rev- or Tat-specific antibodies.

Table 2
Follow-up study of Nef, Rev and Tat gene gun immunised mice[a]

	Months				
	2	3	4	5	6
Nef response					
Ab	2/2	2/2	2/2	2/2	1/2
CTL	0/2	2/2	2/2	1/2	1/2
Proliferation	2/2	2/2	2/2	2/2	1/2
Rev response					
Ab	0/2	0/2	0/2	0/2	0/2
CTL	1/2	2/2	0/2	1/2	0/2
Proliferation	1/2	1/2	1/2	1/2	1/2
Tat response					
Ab	0/2	0/2	0/2	0/2	0/2
CTL	1/2	0/2	2/2	1/2	1/2
Proliferation	2/2	0/2	0/2	0/2	0/2

[a] Shown is the amount of responding mice/tested mice.

Table 1
Immune responses in DNA-immunised mice (measured 4 weeks after last immunisation)[a]

Response detected	Plasmid and method used for immunisation				
	Nef GG	Nef IM	Nef ID	Rev GG	Tat GG
Ab	23/23	6/12	9/12	0/13	0/13
CTL	13/23	5/12	8/12	8/13	2/13
Proliferation	13/13	6/8	5/8	13/13	1/9

[a] Shown is the amount of responding mice/tested mice.

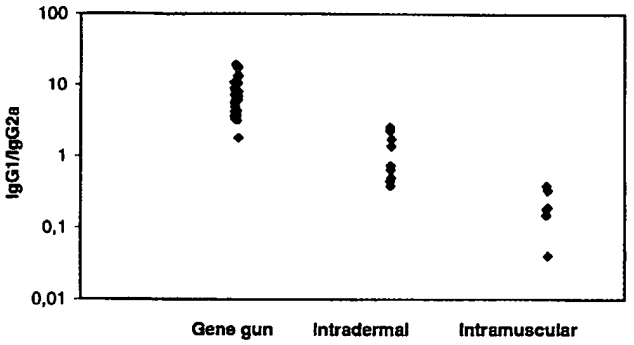

Fig. 3. IgG1/IgG2a antibody subclass ratio in sera of mice immunised with various DNA-delivery methods.

Epitope characterisation of antibodies raised with various immunisation methods was performed in order to see whether various immunisation routes would result in differences in antigen processing, presentation and humoral response. Three sera from each immunisation group giving high EIU values against whole Nef protein (titers 1:5000–1:10 000) were selected for epitope mapping. Table 3 shows the EIUpep values for each peptide and each serum; these values reflect the intensity of colour produced by antibodies binding to Nef peptides as compared to binding to whole Nef protein. With some sera, peptides were recognized even better than the whole protein probably due to steric hindrance caused by other portions of the protein. As seen from Table 3, the N-terminus of Nef contains two antigenic regions which are recognized by almost all antibodies irrespective of the immunisation method. These areas lie between amino acids 9–24 (SVIGWLTVRERMRRAE) and amino acids 49–64 (AATNAACAWLEAQEEE). In addition, peptide 118–133 (QGYFPDWQNYTPGPGV) was found to be considerably antigenic and occasional reactivity against several other peptides was detected. Interestingly, even though all the sera belonging to the gene gun group showed clear humoral response against the whole Nef protein, only one out of three recognized well linear epitopes represented by peptides.

3.3. Cell-mediated immune responses in vaccinated mice

The Nef-plasmid induced an antigen-specific CTL response in 57% of the gene gun immunised mice and all animals tested also had a proliferative response against Nef (Table 1). The magnitude of positive CTL response in these animals varied between 6 and 22% specific lysis (mean 11%), when background lysis of vaccinia wt infected target cells was below 2%. The stimulation index in these mice varied between 3 and 41 (mean 12). pBN-Nef was also immunogenic when given intramuscularly or intradermally: 42% of the Nef IM group and 67% of the Nef ID group had a CTL response against Nef, and 75% of the Nef IM group and 63% of the Nef ID group showed proliferation

Table 3
Epitope characterization of Nef-specific antibodies using three different sera from Nef GG group, Nef ID group and Nef IM group[a]

Amino acid position	GG1	GG2	GG3	ID1	ID2	ID3	IM1	IM2	IM3
1–16	0	0	0	2	0	0	0	0	0
9–24	0	0	**54**	**16**	**18**	**56**	**24**	4	**103**
17–32	0	0	0	5	0	0	0	0	0
25–40	2	0	**11**	5	0	**40**	0	0	**13**
33–48	0	0	2	2	0	6	0	0	8
41–56	0	3	5	6	2	6	5	0	5
49–64	0	0	**63**	**14**	**13**	**80**	**19**	**32**	**84**
57–71	0	0	5	0	0	**14**	1	0	**22**
65–80	1	4	1	4	0	**12**	3	0	8
72–88	0	0	3	4	2	7	5	0	**12**
81–96	1	0	0	4	2	0	5	2	0
88–103	2	1	0	6	2	8	6	5	**12**
96–112	3	1	8	7	4	**16**	**12**	4	9
107–123	1	1	**25**	4	3	**21**	**10**	6	7
118–133	0	**10**	0	6	**24**	**18**	2	4	**160**
126–141	2	1	0	7	3	3	4	6	0
134–149	0	0	0	4	**14**	0	0	1	**11**
143–158	1	0	0	5	5	4	4	2	6
151–166	1	**10**	0	9	3	0	8	5	0
159–174	0	5	**13**	0	**27**	**48**	0	0	**109**
167–182	0	0	0	0	0	40	7	0	6
175–190	0	0	0	3	1	2	2	0	1
183–198	4	0	0	4	2	5	4	1	0
191–206	7	3	6	6	2	**19**	8	1	8

[a] Shown are the EIUpep values for each peptide. Values above the cut-off limit 10 are shown in bold.

upon stimulation with Nef (Table 1). CTL responses were highest in Nef ID group (variation between 7 and 31%, mean 20%), whereas the best proliferative responses were seen in Nef GG group. Cell-mediated immune response could be seen in mice to the end of the 6 months follow up period (Table 2) with no signs of decreasing efficiency (data not shown).

The pBN-Rev which raised no antibodies was, however, immunogenic when analysed for cell-mediated immune response: 62% of Rev GG group showed CTL-response and all animals tested (13/13) gave a proliferative response against Rev (Table 1). Rev-specific CTL responses in reactive mice varied between 6 and 15% specific lysis (mean 11%) and stimulation index in these mice varied between 3 and 11 (mean 6). Cell-mediated immune responses could be detected up to 5–6 months (Table 2); the proliferation index in these mice remained at the same level but a decrease in the magnitude of CTL response was seen (data not shown).

The pBN-Tat which could not raise humoral response was also poor in raising cell-mediated responses in immunised animals. Only two out of 13 mice showed CTL-response (mean 13%) and one out of nine mice tested a weak proliferative response against Tat. However, during the follow up period CTL-responses were seen for 6 months even though the size of the follow up population (two animals/month) was small (Table 2). No Nef-, Rev- or Tat-specific proliferation or cytotoxicity were seen in control mice.

4. Discussion

This study was designed to further characterize the expression and immunogenicity of a cloned gene delivered in the novel, self-replicating pBN-plasmid, which is a possible candidate for vaccine development. According to our results, the pBN-plasmid is a potent expression vector as only 10 pg of the pBN-Rev plasmid transfected into cells produces functionally active protein. Since there are reports showing that Rev function requires a critical level of intracellular protein expression, and that sequential binding of multiple Rev molecules to the RRE provides an explanation for the observed threshold effect for Rev function [26,27], the tests showing clearly detectable Rev function indicate that Rev is expressed at adequate levels. Furthermore, we previously showed with immunohistochemical staining that pBN-Nef plasmids can express the cloned gene for 3 weeks [17]. Our present results with Tat indicate that the protein expressed by the plasmid is functionally active at least one week after transfection. This prolonged expression is most likely due to the capacity of the plasmid to self-replicate in mammalian cells using the bovine papilloma virus derived replication machinery.

The pBN-Nef vector is capable of inducing both cell-mediated and humoral immune responses in mice and this effect can be achieved using different immunisation methods. However, even though we showed in in vitro assays that pBN-plasmids carrying either rev- or tat-gene do produce the corresponding functionally active proteins, the in vivo immune response raised by them was different from the one raised against nef, the lack of an antibody response being the most striking difference. These results are consistent with previous DNA vaccination studies with a CMV-based vector, where it was shown that in the sera of Balb/c mice immunised with HIV-1 regulatory genes, antibodies against Nef were found frequently but antibodies against Rev and Tat were almost absent [5].

There may be several reasons for the lack of an antibody response against Rev and Tat. Firstly, small quantities of antigen (< 1 ng) are sufficient to elicit cellular responses in genetic immunisation whereas at least a 40-fold higher amount of the same antigen is required to elicit a high titer antibody response [28]. In the case of our plasmids, the Nef-construct may be superior to Rev- and Tat-constructs in this respect. Secondly, natural Rev and Tat proteins are less immunogenic than Nef also in HIV-infected individuals. One explanation for this may be the different localisation of these three proteins within the cell. Rev and Tat are mainly located in the nucleus [11] while Nef binds to the cellular membranes and is expressed on the surface of infected T-cells [29,30]. Taken together, the reason for lack of antibodies in Rev and Tat immunised mice is unclear, but as a cell-mediated response is desirable in the case of such intracellular pathogens as HIV, antibodies play a minor role in immune defence.

T-cell responses against Nef and Rev were found in most of the immunised mice, which is consistent with the previous study [5]. Contradictory to our results, other groups have been able to raise T-cell responses to Tat in Balb/c mice [5,8]. This difference may be due to a lower expression level of our pBN-Tat plasmid or to differences in immunisation protocols. In one study, where immune response towards Tat was shown, the total amount of DNA delivered intramuscularly was 450 μg [8], which is much higher than the amount used in our study (6 μg with gene gun). In addition, as Tat is shown to be a potent immunosupressor [31–33] the lack of detectable responses seen in our Tat-immunisation studies may also be a consequence of this feature of the protein.

In various immunisation methods different cells may be responsible for taking up the injected plasmid, and antigen processing and presentation in these cells may vary leading to humoral and cellular immune responses towards different epitopes. In this study we showed that whether the pBN-Nef plasmid is delivered by gene gun or is injected through intradermal or intramuscular

route, the antibodies directed against Nef recognise two main epitopes between amino acids 9–24 and 49–64 (SVIGWLTVRERMRRAE and AATNAACAWLEAQEEE, respectively). These epitopes overlap with some of the epitopes recognised by sera of HIV-infected individuals [34] and by mice immunised with corresponding whole protein [29]. Previous epitope mappings in DNA-immunised animals showed that the C-terminus of Nef is the most immunogenic [5,7]. We also detected some reactivity against amino acids 159–174, 167–182 and 191–206, but the epitopes situated at the N-terminus and in the core region of Nef were more frequently recognised.

In this study the antibody subclasses were characterized in order to see whether any of the immunisation routes would favour either Th type 1 or 2 response. The results of antibody subclass analysis divided the responses into separate Th1 (IM) and Th2 (GG) pathways, and the detected IgG1 and IgG2a subclass pattern stayed stable during the whole follow-up period. This is consistent with previous findings [35] where it was shown that in DNA immunisation the antibody subclass pattern is fixed already at the primary immunisation and can not be modulated even by successive immunisations using other methods.

As our pBN-plasmid is capable of replicating within the cell, we also wanted to assess the safety of the vector. Therefore, we used vector-specific primers and PCR as well as Southern blotting to analyze the spreading of plasmid into various tissues in animals immunised with different methods. No signs of plasmid were seen in any tissue analyzed by these methods, nor did we see any pathological lesions in histological sections taken from these tissues (data not shown). In order to detect anti-vector responses, which might have been induced by the extensive replication of the vector or by the several inoculations given to mice, we also looked for BPV E2 antibodies in immunised animals; E1 antibodies were not tested due to the lack of purified antigen. No E2 specific antibodies were seen in Western blots (data not shown). E1 and E2 do not belong to the oncogenic proteins of BPV.

Overall, we have confirmed that the pBN-plasmid is capable of inducing an immune response with various DNA delivery methods in mice, but studies of the efficacy of this vector in other animals including non-human primates are necessary. We also showed that both Nef and Rev are good candidates for an HIV-1 vaccine, in which a cell-mediated immune response is desirable. In order to include Tat in vaccines, modifications improving the antigenicity and diminishing the immunosuppressive [31–33] and pathological effects of Tat [36–38] may be necessary.

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